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## Coaggregation of oral bacteria. A physicochemical study based on microcalorimetry

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### *Document Version*

Publisher's PDF, also known as Version of record

### *Publication date:*

2005

[Link to publication in University of Groningen/UMCG research database](#)

### *Citation for published version (APA):*

Postollec, F. (2005). Coaggregation of oral bacteria. A physicochemical study based on microcalorimetry. Groningen: s.n.

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## Summary

Coaggregation is defined as the specific recognition and interaction between bacteria in suspension. It was first reported in 1970 by Gibbons & Nygaard and has been mostly described between bacteria isolated from human dental plaque. Since the last ten years, coaggregation has been recognized amongst bacteria isolated from freshwater ecosystems, human and animal gastrointestinal and urogenital tracts. Therefore, it is becoming increasingly clear that coaggregation is likely to be a widespread phenomenon playing a crucial role in the development of multi-species bacterial communities called biofilms. A common property of coaggregation in all these environments appears to be the specific recognition between complementary cell-surface molecules. Visual, spectrophotometric, microscopic and radioactivity based assays have been developed in order to access binding affinity between bacterial cells (**chapter 1**). Because hydrodynamic and mass transport conditions between bacteria in suspension are difficult to control in practice, most of the techniques used to study coaggregation are at best semi quantitative. Mechanistically, coaggregation has been described as resulting from attractive Lifshitz-Van der Waals and acid-base interactions, while coaggregation occurs despite repulsive electrostatic forces. However, several aspects of its mechanism, like the exact influence of temperature on coaggregation, are still poorly understood.

In contrast to the thermodynamic approach, where a free energy change of coaggregation is calculated from *ex situ* contact angle measurements, microcalorimetry directly measures heat flow upon interaction in suspension. It is surprising that isothermal titration calorimetry (ITC) has never been used to access thermodynamic binding profiles between cells. Therefore the aim of this thesis is to propose a method based on microcalorimetry to measure the enthalpy of interaction between whole bacterial cells in suspension in an attempt to further understand the coaggregation phenomenon. To that end, one bacterial suspension is injected to its partner suspension applying a controlled mixing procedure. Measurements are done for a coaggregating pair (*Actinomyces naeslundii* 147 / *Streptococcus oralis* J22) and a non-coaggregating oral bacterial pair (*A. naeslundii* 147 / *Streptococcus sanguis* PK1889) used as a control. The approach presented is multiple, i.e. microcalorimetric measurements are complemented with determination of binding isotherms, confocal laser scanning microscopy (CLSM) and atomic force microscopy (AFM).

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**Chapter 2** describes how ITC can be employed to determine the enthalpy of coaggregation between two oral bacterial pairs. For most biological processes, the enthalpy tends to reach a minimum value, reflecting the most stable state, which is directly related to the heat content of the system. The calorimeter consists of four measuring units where reaction ampoules are filled with 1.5 ml of an *Actinomyces naeslundii* 147 suspension, while reference ampoules are filled with buffer only. After equilibration at 25°C, 80 µl of a streptococcal suspension was titrated into the reaction ampoules. In order to study possible saturation of the binding sites on the actinomyces surface, three consecutive injections with streptococcal suspensions were done. Following each injection, a 20 µl aliquot was taken from the ampoule kept outside the calorimeter and the number of free ( $S_f$ ) and bound ( $S_b$ ) streptococci was determined microscopically. The coaggregation enthalpy was exothermic, i.e. heat was released in the reaction ampoule upon interaction. The heat released by the coaggregating pair minus the heat released by the non-coaggregating pair yielded a coaggregation enthalpy of  $-0.015 \times 10^{-6}$  mJ/bound streptococcus for the first injection. Upon consecutive injections, the coaggregation enthalpy decreased to  $-0.4 \times 10^{-9}$  mJ/bound streptococcus.

In **chapter 3**, titration microcalorimetry is complemented with other techniques to investigate the driving force and pathway-dependency of coaggregation. To that end, the macroscopic turbidity of the bacterial suspension, the morphology of the coaggregates, binding isotherms and heats of interaction were compared between the above-mentioned coaggregating and non-coaggregating pair. The coaggregating pair forms large aggregates, which rapidly sediment from the suspension while the non-coaggregating pair forms only very small coaggregates that remain homogeneously suspended. Coaggregation is further characterized by a high affinity between the partner cells that bind to each other in a strong cooperative mode. The interactions between both pairs occur under the release of heat and are thus enthalpically favorable. More heat is released for the coaggregating than for the non-coaggregating pair. Adding the coaggregating bacteria in steps to each other leads to saturation of enthalpically favorable binding sites. This is observed when the streptococcus is added to the actinomyces as well as when the addition is done the other way around. It is concluded that the cooperativity of the coaggregation process is based on an increase in entropy. It is furthermore shown that the density of the coaggregates as well as the heat effect of their formation depend on the number of steps in which the partner cells are added to each other. Adding *S. oralis* J22 in three steps to *A. naeslundii* 147 results in the formation of denser coaggregates under the release of less heat, as compared to addition in one step. These differences point to a larger entropy increase when in

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a stepwise mixing the coaggregating bacteria are allowed to form more densely packed coaggregates.

The influence of temperature on coaggregation is described in **chapter 4**. Binding isotherms and heats of interaction have been determined at 15, 25 and 40°C for a coaggregating and a non-coaggregating oral bacterial pair. Heats of interaction were measured upon three consecutive injections of streptococci into an actinomyces suspension using isothermal titration calorimetry. After each injection, the number of streptococci injected remaining free in suspension was quantified microscopically and binding isotherms were determined. The binding isotherms for the coaggregating pair show positive cooperative binding. The highest cooperativity, at 25°C, correlates with a strong, macroscopically visible coaggregation. The non-coaggregating pair shows low cooperativity and lacked macroscopically visible coaggregation. Interactions between the coaggregating partners seem to be mainly due to specific, enthalpically saturable and favorable binding sites. Even though the enthalpic part of the interaction is saturated, cooperativity increases with consecutive injections, implying that the coaggregation phenomenon is driven by entropy gain. The change in heat capacity ( $\Delta C_p$ ) is positive for the non-coaggregating pair from 15-40°C as well as for the coaggregating pair beyond 25°C. At lower temperatures the coaggregating pair causes a negative  $\Delta C_p$ . The decrease in heat capacity together with an increase in entropy is considered to be indicative of hydrophobic interactions playing an important role in the formation of large coaggregates as observed for the coaggregating pair at 25°C.

Interaction forces between coaggregating and non-coaggregating oral bacterial pairs have been investigated in **chapter 5** at 10, 22 and 40°C in buffer with the use of AFM. *A. naeshundii* 147 was immobilized on poly-L-lysine coated tipless AFM cantilevers, while streptococci were immobilized on poly-L-lysine functionalized glass surfaces. Upon approach of the actinomyces probe to the top of a streptococcal cell, a repulsive force was measured at all three temperatures and regardless of whether a coaggregating or non-coaggregating pair was involved. However, upon retraction, the coaggregating pair exhibited significantly larger maximal adhesion forces and adhesion energies than the non-coaggregating pair. For the coaggregating pair, the adhesive interaction was smallest at 40°C.

As suggested in the **general discussion**, oral bacteria have developed adhesive abilities which are differently modulated by environmental conditions in order to respond and prosper in a dynamic environment such as the oral cavity. Coaggregation is the result of a complex interplay between specific, short-range interactions and non-specific, non-saturable long-range interactions.

## *Summary*

ITC, as demonstrated for the first time in this thesis, offers the possibility to measure such interactions between whole cells in suspension. In view of the complexity of the system involving two bacterial entities, ITC has been complemented by binding isotherms, visual coaggregating score, CLSM, AFM. The collection of techniques described in this thesis offers a promising approach to study coaggregation or other cellular interaction based on microcalorimetry. Bacterial interactions (with other cells or with surfaces) are crucial in order to comprehend the early stages of development of multi-species communities. Therefore some suggestions are given for further research on the widespread phenomenon of biofilms.